Epigenetic editing

Cano Rodriguez, David

CHAPTER 5

Targeting two different promoters of endogenous RASSF1 to confirm its dual role in cancer

CHAPTER 5

Targeting two different promoters of endogenous RASSF1 to confirm its dual role in cancer

In progress

David Cano-Rodriguez1, Michael Eyres2, Marcel HJ Ruiters1, Eric O'Neill2 & Marianne G. Rots1
1Epigenetic Editing Research Group, Department of Pathology and Medical Biology, University of Groningen, University Medical Centre Groningen, Hanzeplein 1, 9713GZ, Groningen, The Netherlands
2Cell Signaling Group, Department of Oncology, CRUK/MRC Oxford Institute, University of Oxford, Oxford OX3 7DQ, UK

Abstract

Epigenetics determines the accessibility of chromatin and underlies gene transcription programming, including usage of alternative transcription start sites. Diseases frequently exhibit aberrant patterns of epigenetic modifications. On one hand, DNA hypermethylation is often observed at gene promoters and is thought to promote e.g. tumorigenesis by the silencing of tumor suppressor genes. In the same way, active histone marks are associated with the overexpression of oncogenes. In order to avoid cell cycle control checkpoints, cancer cells make use of these two epigenetic mechanisms. Here we assessed the role of the differential promoter methylation of the tumor suppressor gene RASSF1. RASSF1 is controlled by two main distinct promoters yielding two different transcripts with contrasting effects on cellular functions (transcript A is tumor suppressive; C is oncogenic). Using the CRISPR-dCas9 system or Zinc Finger Proteins fused to transcriptional modulators to silence or activate the different promoters of RASSF1, we were able to confirm previous findings: RASSF1a overexpression shows a tumor suppressive role and induction of RASSF1c resulted in increased levels of OCT4 and Nanog. Since epigenetic factors are reversible, they provide promising new alternatives as therapeutic targets. By using epigenetic editing as a targeted approach, we are able to circumvent the limitations of current epigenetic drugs due to lack of locus-specificity.

Introduction

The RASSF family of proteins is comprised of ten members each with multiple splice variants. These proteins were named due to the presence of a Ras association (RA) domain in their N-terminus or C-terminus. The RA domain potentially interacts with the Ras GTPase family of proteins that control a number of cellular processes including membrane trafficking, apoptosis, and proliferation[1-3]. The RASSF1 gene is one of the most studied genes of the family. The gene is located on the small arm of chromosome 3 (3p21.3) and loss of heterozygosity studies identified loss of this chromosomal region in various tumors, suggesting the presence of tumor suppressor genes[4-6]. Indeed, RASSF1 is currently considered an important tumor suppressor gene for various tumors. The gene codes for eight exons and generates seven tissue-specific transcripts (RASSF1A-G) by differential promoter usage and alternative splicing. The two major forms, RASSF1A and RASSF1C, are transcribed from two distinct CpG island containing promoters, separated by approximately 2000 base pairs. Both forms are ubiquitously expressed in normal tissues. The resulting mRNAs differ primarily in the selection of the first exon. RASSF1A contains an amino-terminal cysteine-rich region, which is similar to the diacyl glycerol binding domain (C1 domain) found in the protein kinase C family of proteins, and a carboxy-terminal putative Ras-association (RA) domain. RASSF1C is a smaller protein that lacks the amino-terminal C1 domain[1,7,8].

RASSF1A is a component of key cancer pathways, namely Ras/PI3K/AKT, Ras/RAF/MEK/ERK and Hippo pathways. Indeed, inactivation of RASSF1A contributes to pathogenesis and progression of solid tumours[9-18]. The Hippo pathway, for example, is a developmental conserved pathway that regulates cell proliferation and differentiation to specify organ size.

In line with the developmental role, loss of Hippo pathway components have been widely associated with increased spontaneous tumor formation in model systems [19,20]. Moreover, deregulation of the pathway has been identified in a broad range of human carcinomas including lung, breast, colorectal, pancreatic, ovarian and liver, but genetic events driving total inactivation of the pathway have not been found. Epigenetic inactivation of hippo kinase control components, such as RASSF1A, however, are widespread in human tumors and, loss of the Hippo pathway leads to tumors that have a poor overall prognosis for survival[21]. Alternatively, RASSF1A induces apoptosis through the transcriptional regulator Yap1[22], which has been shown to be vital for the survival of both mutant KRAS[23,24] and β -Catenin driven tumors[25]. As RASSF1A is one of the most frequently epigenetically inactivated tumor-suppressor genes in sporadic human malignancies, it provides an promising therapeutic target for transcriptional upregulation.

On the other hand, the expression of the short isoform of RASSF1C promotes breast and lung cancer cell proliferation [26]. In addition, RASSF1C over-expression (and not RASSF1A over-expression) in human cancer cells enhances accumulation of the β-Catenin oncogene, a key player in the Wnt signaling pathway, leading to increased transcriptional activation and cell proliferation [27].It has also been previously shown that over-expression of RASSF1C up-regulates the expression of PIWIL1, a stem cell self-renewal gene [28,29]. Interestingly, methylation of the RASSF1A promoter is associated with expression of the oncogenic isoform, RASSF1C, which can actively drive tumorigenesis and metastasis[30]. Therefore, epigenetic inactivation of the RASSF1A promoter and activation of the downstream RASSF1C promoter plays an important role in a number of distinct tumor types at various tumor stages. Although most of the studies have used conventional overexpression or RNA interference techniques to evaluate the role of these proteins in cancer, endogenous gene expression modulation has never been tested. The influence of epigenetic variations on regulation of transcription and translation is gaining increased attention as modulatory events that are guiding tumor development alongside genetic mutations[31-34]. Despite this, exactly how these epigenetically regulated events are triggered remains unknown and direct evidence for their consequence on tumor development has been lacking.

An elegant method to study functional consequences of local epigenetic changes is epigenetic editing: By fusing DNA targeting domains to epigenetic modulators, any given region in the genome can be targeted and the gene expression patterns can be reprogrammed[35-38]. This has emerged as a powerful tool in investigating the epigenetic events that occur during tumorigenesis, and could also have clinical applications through the silencing of oncogenes or the reactivation of tumor suppressor genes that have been epigenetically silenced[35,38-44]. Here we take this one step further and use engineered Zinc Finger Proteins (ZFPs) and the CRISPR-dCas9 system to target either promoter of RASSF1 to address the effects of endogenous gene expression reprogramming of either transcript. We established an experimental approach to addres the influence of each transcript in cellular behavior and function validating previous findings: RASSF1A has tumor suppressive activity while upregulation of RASSF1C could induce some expression of pluripotency genes such as OCT4 and Nanog.

Materials and methods

Cell culture

Human embryonic kidney cells HEK-293T, MCF7 and MDA-MB-231 breast cancer cells, HeLa cervical cancer cells, SKOV3 ovarian cancer cells, Hop92 lung cancer cells, HT-29 colon cancer cells and U2OS osteosarcoma cells were obtained from ATCC and cultured in DMEM (BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 50 μg/ml gentamycine sulfate. Cells were cultured in a humidified atmosphere at 37° C supplemented with 5% CO2.

CRISPR-dCas9 plasmid construction and engineering ZFPs

Plasmids containing a mammalian codon-optimized dCas9-VP64 activator (pMLM3705: Addgene #47754) and the single-chain guide RNA encoding plasmid (pMLM3636: Addgene #43860) were kindly provided by Keith Joung. As described previously, an additional multiple cloning site was added by replacing the VP64 activator in the dCas9-VP64 with a sequence containing a PacI restriction site (new plasmid referred to as dCas9-Empty)[45].

The Super Krab Domain (SKD) was subcloned from pMX-6ZF-SKD43 into dCas9-VP64, by using BamHI and XhoI enzymes, to replace VP64 with SKD. Four target regions of 20 bps of the RASSF1C promoter were selected to design gRNAs based on close proximity to the transcription start site (TSS) (gRNA1: TTGTGCGCTTGCCCGGACGC; gRNA2: CGGAGCGATGAGGTCATTCC; gRNA3: GGATCTAGCTCTTGTCTCAT reverse strand; gRNA4: AGTGCGCGTGCGCGGAGCCT reverse strand). Cloning of gRNAs was achieved as previously described[45]. Briefly, pairs of DNA oligonucleotides encoding 20 nucleotide gRNA targeting sequences were annealed together to create double-stranded DNA fragments with 4-bp overhangs. These fragments were ligated into BsmBI-digested plasmid pMLM3636. Artificial transcription factors targeting the RASSF1A promoter were reported previously[45] and selected based on high affinity predictions (www.zincfingertools.org) and the uniqueness of the target sites confirmed by a blast on NCBI (ZFX: GGAGGGGACGAAGGAGGG; ZFY: CGCAGAGCCCCCCCCGCC reverse strand; ZFZ: GGCGCTGAAGTCGGGGCC).

Retroviral transductions

HEK293T cells were co-transfected with the retroviral vector pMX-IRES-GFP along with VSV-G viral envelope (pMD2.G) and the gag/pol proteins (pMDLg/pRRE) as described previously[43] using CaPO4. 48 and 72 hours after transfection, the viral supernatant was used to transduce host cells supplemented with FBS and 5 μg/ml polybrene (Sigma, St. Louis, MO, USA). Cells were harvested for further experiments three days after the last transduction. GFP positivity of cells was assessed on a Calibur Flow Cytometer (Beckton Dickenson Biosciences).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Scientific, Leon-Rot, Germany) according to protocol. Subsequently, cDNA was synthesized with random hexamer primers using the Revertaid cDNA synthesis kit (Thermo Scientific). qRT-PCR was executed using 10 ng of cDNA. We assessed the expression of target genes using ABsolute qPCR SYBR Green (Thermoscientific) and specific primers (Table 1). All reactions were done in triplicate per sample and averaged from at least 3 independent experiments. In order to ensure a signal with the qRT-PCR also for low expressed genes, we run the PCR for 45 cycles. CT values were acquired for all samples, allowing quantitative analysis. Fold change in mRNA expression above control untreated cells was calculated based on the cycle threshold ($\Delta\Delta$Ct) method after normalization to GAPDH expression.

Clonogenic assay

Following transduction, cell were plated in 6-wells plates (2000-4000 cells per well). After two weeks, medium was aspirated and colonies were stained with Coomassie brilliant blue (Bio-Rad). The number of colonies was determined using phase contrast microscopy and Image J.

Apoptosis assay

Cell apoptosis was measured using the 1, 1",3,3,3",3"-Hexamethylindodicarbocyanine iodide (DilC) assay (Enzo Life Sciences) and the Annexin V-PI assays (Sigma) according to the manufacturers protocol. For the DilC assay, following treatment, cells were trypsinized and incubated in culture medium supplemented with DilC (50 nM) for 15 min at 37°C. After washing with PBS, DilC signal was analyzed using FACS Calibur (BD Biosciences).

The percentage of apoptotic cells was determined as the number of viable cells with decreased DilC intensity, as reported before[42]. Assays were performed in 96-well plates according to the manufacturers instruction. Each experiment was carried out in triplicate and averaged from at least 3 independent experiments.

Statistics

Statistical tests were performed using the Graphpad Prism 5 software (GraphPad Software). All experiments were performed at least three times, unless stated otherwise. Relevant comparisons were evaluated by unpaired, two-tailed t-test. A P value of <0.05 was considered statistically significant. All data are presented as the mean ± sem.

Results

Endogenous RASSF1A upregulation induces cell death and decreases proliferation

To study the role of endogenous RASSF1A, we selected two cell lines with repression of RASSF1A due to promoter hypermethylation (MCF7 and SKOV3), and two cell lines with RASSF1A expression (HT-29 and Hop92). Since hypermethylated CpG islands have been shown to pose a barrier to dCas9 targeting[45], we decided to use ZFPs to target the promoter of RASSF1A. By retroviral delivery of three different zinc finger proteins (ZFX, ZFY, ZFZ; Fig. 1a), we addressed the gene expression modulation using a transcriptional activator (VP64) in the four cell lines (Fig. 1a). One of the zinc fingers (ZFZ) was able to significantly upregulate the expression of RASSF1A in SKOV3 and HT-29. The upregulation of RASSF1A gene was followed by an increase in apoptosis, as measured by DilC staining, also for MCF7 (Fig. 1b). To assess whether upregulation of RASSF1A results in cell growth inhibition, we performed a clonogenic assay to address the capacity of the cell lines to form colonies (Fig. 1c,d). Compared to untreated cells, SKOV3 and HT-29 cells treated to express ZFX-VP64 or ZFZ-VP64 showed less capacity to form colonies. For ZF Z-VP64, colony formation was reduced more than two-fold, also for MCF-7.

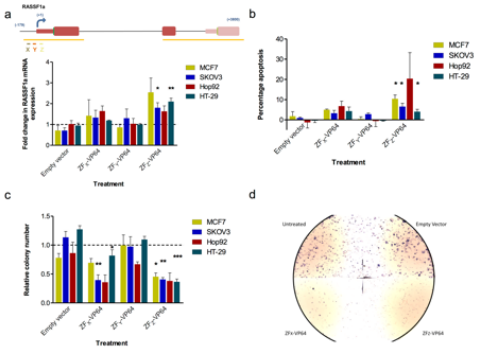

Figure 1. *RASSF1A activation by means of endogenous gene-targeting with zinc finger proteins in four cell lines. a) Activation of endogenous RASSF1A in four cancer cell lines (two hypermethylated cell lines MCF7 and SKOV3, and two cell lines with active RASSF1A, Hop92 and HT-29); representation of the RASSF1 gene promoter, transcription start site (TSS), yellow bars represent the CpG islands and X, Y and Z represent the region targeted. b) Apoptosis assay measured by DilC staining in cells treated with different ZFs after RASSF1A upregulation. c) Colony-forming assay in cells after RASSF1A upregulation. d) Visual representation of the colony-forming assay from HT-29 cells. (two-sided unpaired t-test, *P < 0.05, **P < 0.01, ***P < 0.001 n = 3 independent experiments; error bars, s.e.m.).*

Downregulation of RASSF1A using a transcriptional repressor

To assess the effects of RASSF1A downregulation, we fused the engineered ZFPs to a transcriptional repressor Super KRAB Domain (SKD). By targeting the promoter of the active RASSF1A in Hop92 and HT-29 cancer cells, we were able to efficiently repress the gene by using ZFX (2-fold for Hop92 and 3-fold for HT-29) (Fig. 2).

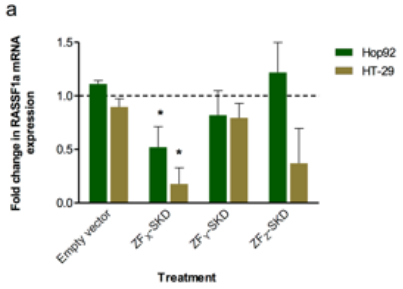

Figure 2. *RASSF1A repression by means of endogenous gene-targeting with zinc finger proteins in two cell lines. a) Repression of endogenous RASSF1A in two cancer cell lines with active expression, using the Super Krab Domain (SKD) repressor. (two-sided unpaired t-test, *P < 0.05, n = 3 independent experiments; error bars, s.e.m.).*

This downregulation was followed by changes in cellular behavior, while the cells treated with empty vector or other ZFPs showed no difference, cells transduced to express the ZFX -fusion had a more stem cell-like phenotype, forming non-adherent tumor sphere colonies and exhibiting epithelial to mesenchymal transition (EMT) (data not shown).

Changes in RASSF1C expression has different outcomes depending on RASSF1A status

In order to target the promoter of RASSF1C, we made use of the CRISPR-dCas9 system, since most of the cancer cells have active open chromatin at this locus, in contrast to RASSF1A (see Fig. 3a for MCF7 and HeLa). We designed four different gRNAs to target the promoter of RASSF1C, with at least 40 bps of distance between each other (Fig. 3B). To test the gene expression modulation, we used two cell lines that differ on the status of RASSF1A expression: HeLa cells have expression of endogenous RASSF1A, while MCF7 cells have hypermethylation of RASSF1A promoter, hence no expression[22,30]. In MCF7 cells, significant upregulation of RASSF1C was observed using dCas9 fused to VP64 and significant downregulation when using dCas9-SKD (Fig. 3c). Remarkably, when targeting HeLa cells, we were able to upregulate the expression of RASSF1C by targeting the promoter with dCas9, dCas9-SKD or dCas9-VP64. This observation requires further in depth study. To confirm the upregulation seen in MCF7, we assayed additional cell lines that have no expression of RASSF1A, (Fig. 3d). Upregulation of RASSF1C was observed in the three additional cell lines (HEK293T, U2OS and MDA-MB-231).

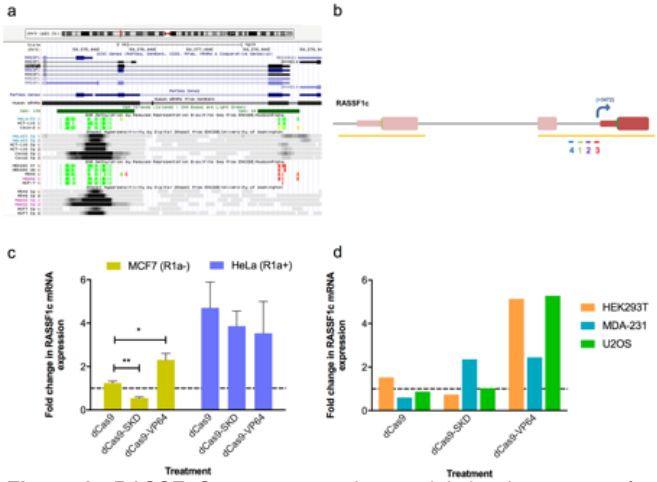

Figure 3. *RASSF1C gene expression modulation by means of endogenous gene-targeting with the CRISPR-dCas9 system in different cell lines. a) Epigenetic landscape of the two promoters from the RASSF1 gene in two cell lines with distinct patterns of RASSF1 expression transcripts from ENCODE (MCF7: 1A negative, 1C positive; HeLa: 1A positive, 1C positive). Dark green bars represent the CpG islands; light green (unmehtylated), yellow (partially methylated) or red (hypermethylated) bars represent DNA methylation profiles; black represent the H3K4me3 peaks of HeLa and MCF7. b) Representation of the RASSF1C gene promoter, transcription start site (TSS), yellow bars represent the CpG islands and 1, 2, 3 and 4 represent the region targeted with sgRNAs. c) Gene expression modulation of the RASSF1C using dCas9, the repressor dCas9-SKD and the activator dCas9-VP64, in the two cell lines with different patterns of transcript expression (n = 3 independent experiments; error bars, s.e.m. * P<0,05, ** P<0,01)). d) Gene expression modulation of RASSF1C in 3 cell lines with RASSF1A repression (n = 1 independent experiment).*

Endogenous RASSF1C upregulation affects downstream targets

To address whether RASSF1C upregulation triggered a biological response, we decided to first analyze the expression of candidate downstream targets genes. When using the MCF7 transfected cells, some upregulation of RASSF1A was observed, but no clear difference on additional downstream targets (Fig. 4a). Moreover, we tested the expression of stemness markers (MYC, OCT4, NANOG and SOX2) (Fig. 4b). OCT4 and NANOG expression seemed upregulated upon RASSF1C overexpression (13,6- and 9,1-fold, respectively). In addition to these markers, we also tested the expression of EMT markers, as they have been linked to RASSF1C expression. We saw no clear overexpression of EMT markers tested in the MCF7 cells with dCas9-VP64-induced RASSF1C expression when compared to cells expressing dCas only or the SKD-fusion (Fig. 4c).

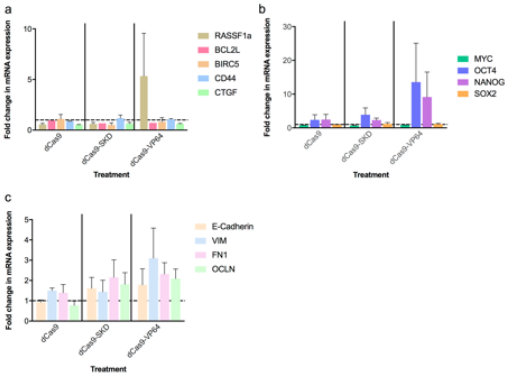

Figure 4. *Gene expression changes in downstream targets of RASSF1C after gene expression modulation in MCF7 cells. a) Changes in gene expression of RASSF1C direct targets and RASSF1A. b) Changes in gene expression of stemness markers. c) Changes in gene expression of EMT markers. (n = 2 independent experiments; error bars, s.e.m.).*

Discussion

Using two different targeting platforms, we were able to show gene expression modulation of the two major transcripts of RASSF1. Outcomes of changes in endogenous gene expression were in line with previous studies using exogenous overexpression, and a dual role for RASSF1 gene is seen depending on the expression of either the A or C transcript. On one hand RASSF1C is thought to promote stemness and cell proliferation: Upon upregulation of endogenous RASSF1C, some indirect upregulation of OCT4 and NANOG, two key transcription factors involved in pluripotency and stemness, was achieved. In contrast to RASSF1A, RASSF1C does not have tumor suppressor properties, but there is increasing evidence suggesting that it functions as an oncogene. Overexpression of RASSF1C in breast and lung cancer cells resulted in enhanced cell migration/invasion[46,47] and it was shown to be overexpressed in pancreatic endocrine tumors[48]. It was also reported that RASSF1C could activate osteoblast cell proliferation through interaction with IGFBP-5[49]. Additionally, over-expression of RASSF1C results in significant accumulation of the β-catenin oncogene, a key player in the Wnt signaling pathway, leading to increased transcriptional activation and cell proliferation[27]; RASSF1C associates with SFCb-TrCP ligase and promotes the accumulation of β-catenin by inhibiting its degradation. Likewise, the same effect was seen when silencing RASSF1A, implying that the balance between the two isoforms is crucial for the bTrCP-mediated degradation of β-catenin[27].

On the other hand, upregulation of RASSF1A exhibits tumor suppressive activity, by inducing apoptosis and inhibiting cell proliferation. Our observations that endogenous activation of RASSF1A, via artificial transcription factors, increases cell death and decreases cell viability are thus supported by the phenotype of tumor cell lines with constitutive overexpression of RASSF1A. The observations in NSCLC, prostate, kidney, nasopharyngeal carcinoma, and glioma cell lines indicate that RASSF1A expressing cells are less viable, growth suppressed and less invasive[4,10,50-53]. This might be related to the key role of RASSF1A in regulating the cell cycle. For instance, RASSF1A inhibits accumulation of cyclin D116 (possibly through JNK kinase pathway [54], suppression of AP-1 activity[55], or both) and blocks the cell cycle at the G1/S-phase transition by interacting with p120E4F , a protein known to associate with pRb, p53 and p14ARF[56-58].

Additionally, RASSF1A regulates apoptosis via at least two pathways: the importance of RASSF1A in death receptor dependent cell death via associations with MOAP-1 (Modulator of apoptosis 1)[59-61], as well as RASSF1A can associate with the Hippo pathway pro-apoptotic kinase, MST1/2 to modulate its kinase activity and promote cell death[22,62,63]. These associations function to prevent excessive growth and allow RASSF1A to function as a tumor suppressor. RASSF1A is also involved in DNA damage response as seen by the apoptotic response to chemotherapeutic agents. RASSF1A enhances the proapoptotic activity of the Hippo pathway and limits oncogenic potential after damage[17,20]. Although the Hippo pathway is a major regulator of proliferation, growth and differentiation, genetic mutations in Hippo pathway components in human cancers are relatively rare[19].
However, methylation of the RASSF1A promoter frequently epigenetically inactivates RASSF1A and can result in an oncogenic isoform switch to RASSF1C, which can actively drive tumorigenesis and metastasis[30]. RASSF1A activation of the Hippo pathway maintains phosphorylation of YAP1, a component of the Hippo pathway. Upon loss of the phosphorylation, YAP1 is permissive for activation but requires additional modifications for nuclear localization and transcriptional transactivation, which is dependent on loss of the RASSF1A transcript and expression of RASSF1C. RASSF1A loss drives RASSF1C-YAP1/β-catenin-mediated transcription and invasion. This is mainly through activation of downstream targets such as MYC and EMT markers. Here we show that activation of endogenous RASSF1C, promotes activation of stemness markers, such as OCT4 and NANOG. Despite harboring 60% amino acid identity, RASSF1A and RASSF1C display distinctive biological properties.

We demonstrate that epigenetic editing and artificial transcription factors are powerful tools to investigate the role of different isoforms from the same gene, and to elucidate the role of each individual transcript. Most of the studies so far make use of RNA interference or cDNA transfection, but these are not adequate to study the mechanisms of isoform balance regulation between different promoters. By using DNA targeting platforms such as Zinc Finger Proteins and CRISPR-dCas9 we were able to target the two different promoters of the RASSF1 gene and to provide insights into the opposing roles of the A and C transcripts. These data open new avenues to investigate how the regulation of these two transcripts is achieved and how this is impaired in cancer. Understanding the switch of one isoform to the other, by hypermethylation and inactivation of a CpG island, allows interfering with proliferation in a more precise way, circumventing unwanted effects such as inducing expression of oncogene isoforms. Current epigenetic drugs make use of general epigenetic enzyme inhibitors, which at the end has genome wide effects. Genome targeting is a precise tool to achieve gene reprogramming in a specific manner.

REFERENCES

1 Dammann, R. *et al.* The tumor suppressor RASSF1A in human carcinogenesis: an update. *Histol Histopathol* **20**, 645-663 (2005).

2 van der Weyden, L. & Adams, D. J. The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochim Biophys Acta* **1776**, 58-85, doi:10.1016/j.bbcan.2007.06.003 (2007).

3 Volodko, N., Gordon, M., Salla, M., Ghazaleh, H. A. & Baksh, S. RASSF tumor suppressor gene family: biological functions and regulation. *FEBS Lett* **588**, 2671-2684, doi:10.1016/j.febslet.2014.02.041 (2014).

4 Dammann, R. *et al.* Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* **25**, 315-319, doi:10.1038/77083 (2000).

5 Sekido, Y. *et al.* Cloning of a breast cancer homozygous deletion junction narrows the region of search for a 3p21.3 tumor suppressor gene. *Oncogene* **16**, 3151-3157, doi:10.1038/sj.onc.1201858 (1998).

6 Lerman, M. I. & Minna, J. D. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer Res* **60**, 6116-6133 (2000).

7 Richter, A. M., Pfeifer, G. P. & Dammann, R. H. The RASSF proteins in cancer; from epigenetic silencing to functional characterization. *Biochim Biophys Acta* **1796**, 114-128, doi:10.1016/j.bbcan.2009.03.004 (2009).

8 Pfeifer, G. P., Dammann, R. & Tommasi, S. RASSF proteins. *Curr Biol* **20**, R344-345, doi:10.1016/j.cub.2010.02.019 (2010).

9 Agathanggelou, A. *et al.* Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene* **20**, 1509-1518, doi:10.1038/sj.onc.1204175 (2001).

10 Burbee, D. G. *et al.* Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* **93**, 691-699 (2001).

11 Dammann, R., Takahashi, T. & Pfeifer, G. P. The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. *Oncogene* **20**, 3563-3567, doi:10.1038/sj.onc.1204469 (2001).

12 Dammann, R. *et al.* Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. *Histol Histopathol* **18**, 665-677 (2003).

13 Agathanggelou, A., Cooper, W. N. & Latif, F. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. *Cancer Res* **65**, 3497-3508, doi:10.1158/0008-5472.CAN-04-4088 (2005).

14 Ahmed, I. A. *et al.* Epigenetic alterations by methylation of RASSF1A and DAPK1 promoter sequences in mammary carcinoma detected in extracellular tumor DNA. *Cancer Genet Cytogenet* **199**, 96-100, doi:10.1016/j.cancergencyto.2010.02.007 (2010).

15 da Costa Prando, E., Cavalli, L. R. & Rainho, C. A. Evidence of epigenetic regulation of the tumor suppressor gene cluster flanking RASSF1 in breast cancer cell lines. *Epigenetics* **6**, 1413-1424, doi:10.4161/epi.6.12.18271 (2011).

16 Shivakumar, L., Minna, J., Sakamaki, T., Pestell, R. & White, M. A. The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol* **22**, 4309-4318 (2002).

17 Hamilton, G., Yee, K. S., Scrace, S. & O'Neill, E. ATM regulates a RASSF1A-dependent DNA damage response. *Curr Biol* **19**, 2020-2025, doi:10.1016/j.cub.2009.10.040 (2009).

18 Thaler, S., Hähnel, P. S., Schad, A., Dammann, R. & Schuler, M. RASSF1A mediates p21Cip1/Waf1-dependent cell cycle arrest and senescence through modulation of the Raf-MEK-ERK pathway and inhibition of Akt. *Cancer Res* **69**, 1748-1757, doi:10.1158/0008-5472.CAN-08-1377 (2009).

19 Harvey, K. F., Zhang, X. & Thomas, D. M. The Hippo pathway and human cancer. *Nat Rev Cancer* **13**, 246-257, doi:10.1038/nrc3458 (2013).

20 Pefani, D. E. & O'Neill, E. Hippo pathway and protection of genome stability in response to DNA damage. *FEBS J* **283**, 1392-1403, doi:10.1111/febs.13604 (2016).

21 Grawenda, A. M. & O'Neill, E. Clinical utility of RASSF1A methylation in human malignancies. *Br J Cancer* **113**, 372-381, doi:10.1038/bjc.2015.221 (2015).

22 Matallanas, D. *et al.* RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. *Mol Cell* **27**, 962-975, doi:10.1016/j.molcel.2007.08.008 (2007).

23 Kapoor, A. *et al.* Yap1 activation enables bypass of oncogenic Kras addiction in pancreatic cancer. *Cell* **158**, 185-197, doi:10.1016/j.cell.2014.06.003 (2014).

24 Zhang, W. *et al.* Downstream of mutant KRAS, the transcription regulator YAP is essential for neoplastic progression to pancreatic ductal adenocarcinoma. *Sci Signal* **7**, ra42, doi:10.1126/scisignal.2005049 (2014).

25 Rosenbluh, J. *et al.* β-Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell* **151**, 1457-1473, doi:10.1016/j.cell.2012.11.026 (2012).

26 Reeves, M. E., Firek, M., Chen, S. T. & Amaar, Y. The RASSF1 Gene and the Opposing Effects of the RASSF1A and RASSF1C Isoforms on Cell Proliferation and Apoptosis. *Mol Biol Int* **2013**, 145096, doi:10.1155/2013/145096 (2013).

27 Estrabaud, E. *et al.* RASSF1C, an isoform of the tumor suppressor RASSF1A, promotes the accumulation of beta-catenin by interacting with betaTrCP. *Cancer Res* **67**, 1054-1061, doi:10.1158/0008-5472.CAN-06-2530 (2007).

28 Reeves, M. E., Firek, M., Chen, S. T. & Amaar, Y. G. Evidence that RASSF1C stimulation of lung cancer cell proliferation depends on IGFBP-5 and PIWIL1 expression levels. *PLoS One* **9**, e101679, doi:10.1371/journal.pone.0101679 (2014).

29 Reeves, M. E. *et al.* RASSF1C modulates the expression of a stem cell renewal gene, PIWIL1. *BMC Res Notes* **5**, 239, doi:10.1186/1756-0500-5-239 (2012).

30 Vlahov, N. *et al.* Alternate RASSF1 Transcripts Control SRC Activity, E-Cadherin Contacts, and YAP-Mediated Invasion. *Curr Biol* **25**, 3019-3034, doi:10.1016/j.cub.2015.09.072 (2015).

31 Iacobuzio-Donahue, C. A. Epigenetic changes in cancer. *Annu Rev Pathol* **4**, 229-249, doi:10.1146/annurev.pathol.3.121806.151442 (2009).

32 Dawson, M. A. & Kouzarides, T. Cancer epigenetics: from mechanism to therapy. *Cell* **150**, 12-27, doi:10.1016/j.cell.2012.06.013 (2012).

33 van Vlerken, L. E., Hurt, E. M. & Hollingsworth, R. E. The role of epigenetic regulation in stem cell and cancer biology. *J Mol Med (Berl)* **90**, 791-801, doi:10.1007/s00109-012-0917-9 (2012).

34 You, J. S. & Jones, P. A. Cancer genetics and epigenetics: two sides of the same coin? *Cancer Cell* **22**, 9-20, doi:10.1016/j.ccr.2012.06.008 (2012).

35 de Groote, M. L., Verschure, P. J. & Rots, M. G. Epigenetic Editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. *Nucleic Acids Research* **40**, 10596-10613, doi:10.1093/nar/gks863 (2012).

36 Jurkowski, T. P., Ravichandran, M. & Stepper, P. Synthetic epigenetics-towards intelligent control of epigenetic states and cell identity. *Clin Epigenetics* **7**, 18, doi:10.1186/s13148-015-0044-x (2015).

37 Cano-Rodriguez, D. & Rots, M. G. Epigenetic Editing: On the Verge of Reprogramming Gene Expression at

Will. *Current Genetic Medicine Reports*, 1-10, doi:10.1007/s40142-016-0104-3 (2016).

38 Thakore, P. I., Black, J. B., Hilton, I. B. & Gersbach, C. A. Editing the epigenome: technologies for programmable transcription and epigenetic modulation. *Nat Methods* **13**, 127-137, doi:10.1038/nmeth.3733 (2016).

39 Rivenbark, A. G. *et al.* Epigenetic reprogramming of cancer cells via targeted DNA methylation. *Epigenetics* **7**, 350-360, doi:10.4161/epi.19507 (2012).

40 Falahi, F. *et al.* Towards sustained silencing of HER2/neu in cancer by epigenetic editing. *Mol Cancer Res* **11**, 1029-1039, doi:10.1158/1541-7786.MCR-12-0567 (2013).

41 Chen, H. *et al.* Induced DNA demethylation by targeting Ten-Eleven Translocation 2 to the human ICAM-1 promoter. *Nucleic Acids Research* **42**, 1563-1574, doi:10.1093/nar/gkt1019 (2014).

42 Huisman, C. *et al.* Functional validation of putative tumor suppressor gene C13ORF18 in cervical cancer by Artificial Transcription Factors. *Mol Oncol* **7**, 669-679, doi:10.1016/j.molonc.2013.02.017 (2013).

43 van der Gun, B. T. *et al.* Bidirectional modulation of endogenous EpCAM expression to unravel its function in ovarian cancer. *Br J Cancer* **108**, 881-886, doi:10.1038/bjc.2013.45 (2013).

44 Huisman, C. *et al.* Prolonged re-expression of the hypermethylated gene EPB41L3 using artificial transcription factors and epigenetic drugs. *Epigenetics* **10**, 384-396, doi:10.1080/15592294.2015.1034415 (2015).

45 Cano-Rodriguez, D. *et al.* Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. *Nat Commun* **7**, 12284, doi:10.1038/ncomms12284 (2016).

46 Reeves, M. E. *et al.* Ras-association domain family 1C protein promotes breast cancer cell migration and attenuates apoptosis. *BMC Cancer* **10**, 562, doi:10.1186/1471-2407-10-562 (2010).

47 Amaar, Y. G. *et al.* Ras association domain family 1C protein stimulates human lung cancer cell proliferation. *Am J Physiol Lung Cell Mol Physiol* **291**, L1185-1190, doi:10.1152/ajplung.00072.2006 (2006).

48 Malpeli, G. *et al.* Methylation-associated down-regulation of RASSF1A and up-regulation of RASSF1C in pancreatic endocrine tumors. *BMC Cancer* **11**, 351, doi:10.1186/1471-2407-11-351 (2011).

49 Amaar, Y. G., Baylink, D. J. & Mohan, S. Ras-association domain family 1 protein, RASSF1C, is an IGFBP-5 binding partner and a potential regulator of osteoblast cell proliferation. *J Bone Miner Res* **20**, 1430-1439, doi:10.1359/JBMR.050311 (2005).

50 Chow, L. S. *et al.* RASSF1A is a target tumor suppressor from 3p21.3 in nasopharyngeal carcinoma. *Int J Cancer* **109**, 839-847, doi:10.1002/ijc.20079 (2004).

51 Dreijerink, K. *et al.* The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis. *Proc Natl Acad Sci U S A* **98**, 7504-7509, doi:10.1073/pnas.131216298 (2001).

52 Hesson, L. *et al.* Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. *Oncogene* **23**, 2408-2419, doi:10.1038/sj.onc.1207407 (2004).

53 Kuzmin, I. *et al.* The RASSF1A tumor suppressor gene is inactivated in prostate tumors and suppresses growth of prostate carcinoma cells. *Cancer Res* **62**, 3498-3502 (2002).

54 Whang, Y. M., Kim, Y. H., Kim, J. S. & Yoo, Y. D. RASSF1A suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression. *Cancer Res* **65**, 3682-3690, doi:10.1158/0008-5472.CAN-04-2792 (2005).

55 Deng, Z. H., Wen, J. F., Li, J. H., Xiao, D. S. & Zhou, J. H. Activator protein-1 involved in growth inhibition by RASSF1A gene in the human gastric carcinoma cell line SGC7901. *World J Gastroenterol* **14**, 1437-1443 (2008).

56 Fajas, L. *et al.* pRB binds to and modulates the transrepressing activity of the E1A-regulated transcription factor p120E4F. *Proc Natl Acad Sci U S A* **97**, 7738-7743, doi:10.1073/pnas.130198397 (2000).

57 Fenton, S. L. *et al.* Identification of the E1A-regulated transcription factor p120 E4F as an interacting partner of the RASSF1A candidate tumor suppressor gene. *Cancer Res* **64**, 102-107 (2004).

58 Rizos, H. *et al.* Association of p14ARF with the p120E4F transcriptional repressor enhances cell cycle inhibition. *J Biol Chem* **278**, 4981-4989, doi:10.1074/jbc.M210978200 (2003).

59 Tan, K. O. *et al.* MAP-1, a novel proapoptotic protein containing a BH3-like motif that associates with Bax through its Bcl-2 homology domains. *J Biol Chem* **276**, 2802-2807, doi:10.1074/jbc.M008955200 (2001).

60 Baksh, S. *et al.* The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to Bax conformational change and cell death. *Mol Cell* **18**, 637-650, doi:10.1016/j.molcel.2005.05.010 (2005).

61 Foley, C. J. *et al.* Dynamics of RASSF1A/MOAP-1 association with death receptors. *Mol Cell Biol* **28**, 4520-4535, doi:10.1128/MCB.02011-07 (2008).

62 Praskova, M., Khoklatchev, A., Ortiz-Vega, S. & Avruch, J. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *Biochem J* **381**, 453-462, doi:10.1042/BJ20040025 (2004).

63 Oh, H. J. *et al.* Role of the tumor suppressor RASSF1A in Mst1-mediated apoptosis. *Cancer Res* **66**, 2562-2569, doi:10.1158/0008-5472.CAN-05-2951 (2006).